REMARKS

Claims 1-11, 21-30, 63, 64, and 69 were subject to examination in the outstanding Office Action. Claims 1-5, 10, 11, 21, 22, 27, 28, 63, 64, and 69 have been amended above. Withdrawn claims 8-9, 12, 15-18, 25-26, 31, 33, 35-36, 38, 41, 46, 48-51, 53, 55, 57-58, 60, 65 and 68 have also been amended. The claims have been amended to clarify the claimed subject matter, to provide greater consistency in the claim language, and to conform the claims to the elected combination of SEQ ID NOs. Support for specific claim amendments will be discussed below in connection with the rejections under § 112, second paragraph. The remaining issues are discussed below in the order raised in the Office Action.

I. Rejections Under § 112, Second Paragraph.

Claims 1-11, 21-30, 64, and 69 stand rejected under 35 U.S.C. § 112, second paragraph, on various grounds of indefiniteness. The individual rejections are addressed below.

Claim 1 stands rejected as "vague and indefinite due to the lack of clarity of the term 'assessing' in lines 1 and 9." (Office Action ¶ 9). Claim 2 is also rejected due to dependency on Claim 1. Claim 1 has been amended above to omit the word "assessing," thereby addressing this rejection. This amendment is merely cosmetic and clarifying in effect and does not narrow the scope of the claim.

Claim 1 also stands rejected as vague and indefinite "due to the lack of clarity of the term 'similarities' in line 9." (Office Action ¶ 10). Claim 2 is also rejected due to dependency on Claim 1. Claim 1 has been amended to recite "degree of similarity" to clarify the claimed subject matter. Applicants assert that the new language, "degree of similarity," is not indefinite because the degree of similarity will correlate with the degree of genetic relationship. It is up to the user to determine what is considered "similar." Further, it is inherent that the higher the degree of similarity between the fingerprints, the closer the genetic relationship. This amendment merely clarifies the claimed subject matter and is not narrowing in effect.

Claims 2, 4, 22, and 64 stand rejected as vague and indefinite "due to the lack of clarity of that which is encompassed by the restriction fragments." (Office Action ¶

11). The Office Action further requests clarification as to "whether the fragments comprise the full sequence of the elected SEQ ID NOs of the claims, or merely a sequence from within the sequence of the SEQ ID NOs." The recited restriction fragments comprise DNA sequences that include the DNA sequences of the elected SEQ ID NOs., *i.e.*, they include at least the entire sequence of the elected SEQ ID NOs. The claim language has also been amended to recite "polymorphic restriction fragments" to clarify the claimed subject matter. This amendment is merely cosmetic and is supported by the specification at p. 13, lines 20-24.

Claim 3 also stands rejected as indefinite for "failing to recite a final process step." (Office Action ¶ 12). Claims 4-11 are also rejected due to dependency from Claim 3. Claim 3 has been amended to recite a final process step. This amendment is merely cosmetic and clarifying in effect and does not narrow the scope of the claim.

Claim 9 also stands rejected as vague and indefinite "due to the lack of clarity in the listed eight primer pairs to be used due to the term 'or' in line 6." (Office Action ¶ 13). Claim 9 has been amended to clarify the claimed subject matter without narrowing the scope of the claim.

Claims 10, 11, 27, and 28 stand rejected as vague and indefinite on the basis that "it is unclear as to what is defined by 'number' and how the number 'corresponds' to the SEQ ID NOs." (Office Action ¶ 14). Claim 29 is also rejected as indefinite due to dependency from Claim 28. Claims 10, 11, 27, and 28 have been amended to recite "set of amplified polymorphic restriction fragments." There is support for this claim language on page 12, lines 1-5, of the specification and in original claims 3, 12, 21, 31, 41, and 53. Claims 10, 11, 27, and 28 have also been amended to omit the word "correspond." This amendment is merely cosmetic and clarifying in effect and does not narrow the scope of the claim.

Claims 21 and 69 stand rejected as indefinite on the basis that "[t]he terms 'similar' (claim 21, line 12) and 'dissimilar' (claim 69, line 9) in claims 21 and 69 are a relative term which renders the claims indefinite." (Office Action ¶ 15). Claims 22-30 are also rejected as indefinite based on their dependency from Claim 21. Claim 21 has been amended to clarify the claim language and to omit recitation of the word

"similar." Further, applicants respectfully submit that the standard for ascertaining the requisite degree of similarity is described at length in the specification, including at page 19, line 31 through page 22, line 27 and page 31, line 24 through page 32, line 27. Additionally, Claim 69 has been amended to omit the word "dissimilar," and to recite that the two plants are "not essentially the same." This amendment is cosmetic, is merely intended to clarify the claimed subject matter, and does not narrow the scope of the claim.

II. The Pending Claims Are Patentable Over Ling et al. in view of Sukhwinder et al., as Defined by Dice and Over Ling et al. in view of Barker et al., as defined by Tullos

Claims 1-7, 10, 11, 21-24, 27-30, 63, 64, and 69 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Ling et al., in view of Sukhwinder et al., as defined by Dice. Claims 1-6, 10, 11, 21-23, 27-30, 63, 64, and 69 stand rejected under 35 U.S.C. §103 as being unpatentable over Ling et al., in view of Barker et al., as defined by Tullos.

A. Legal Standards For Obviousness

The Patent Office has the initial burden under §103 to establish a *prima facie* case of obviousness. *In re Fine*, 837 F.2d 1071 , 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). The Applicants respectfully note that in order to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings in order to arrive at the claimed invention. Second, the prior art must reveal that in making or carrying out the invention, those of ordinary skill would have a reasonable expectation of success. *Noelle v. Lederman*, 355 F.3d 1343, 1352 (Fed. Cir. 2004). Third, the prior art reference (or references when combined) must teach or suggest all of the claim limitations (MPEP § 706.02(j)). The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both

be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The Federal Circuit has articulated the following legal test for obviousness: "The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art. . . . Both the suggestion and the expectation of success must be founded in the prior art, not in the applicant's disclosure." *In re Dow Chemical*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988) (emphasis added). Obviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion, or incentive supporting the combination. *In re Geiger*, 2 USPQ2d 1276 (Fed. Cir. 1987). The mere fact that references can be combined does not render the combination obvious unless the prior art also suggests the desirability of the combination. *In re Fritch*, 23 USPQ 2d 1780 (CAFC 1992). To support combining references, evidence of a suggestion, teaching, or motivation to combine must be clear and particular, and this requirement for clear and particular evidence is not met by broad and conclusory statements about the teachings of references. *In re Dembiczak*, 50 U.S.P.Q.2d 1614, 1617 (Fed. Cir. 1999). The Court of Appeals for the Federal Circuit has also stated that, to support combining or modifying references, there must be particular evidence from the prior art as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed. *In re Kotzab*, 55 U.S.P.Q.2d 1313, 1317 (Fed. Cir. 2000). Furthermore, the invention must be considered as a whole to avoid hindsight reasoning in which the invention is used as a roadmap to piece together prior art components. *Ruiz v. A.B. Chance*, 357 F.2d 1270, 1275 (Fed. Cir. 2004).

The Applicants respectfully contend that the Patent Office has failed to establish a *prima facie case* of obviousness in the present case.

B. The Claimed Methods are not Obvious over The Cited References

i. Rejections over Ling et al., in view of Sukhwinder et al., as Defined by Dice

In rejecting claims 1-7, 10, 11, 21-24, 27-30, 63, 64, and 69, the Office Action states that "[i]t would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention was made to improve the poinsettia cultivar genetic analysis method of Ling et al. and further modify the RAPD procedure used by Ling et al. to the improved method of cultivar analysis using AFLP techniques as per the teachings of Sukhwinder et al. One of ordinary skill in the art would have been motivated to improve the method of genetic analysis used by Ling et al. from RAPD to the AFLP procedure of analyzing genetic relationships and diversity as opposed to RAPD and RFLP." Applicants respectfully disagree.

The Ling et al. reference concerns the use of RAPD techniques to compare the DNA of nine commercial poinsettia cultivars. The Office Action acknowledges that Ling et al. does not teach the use of AFLP to determine genetic relationship or diversity as required by the claims but argues this is an obvious improvement. As addressed in more detail below, the Applicants respectfully submit that it would not have been obvious at the time of invention to use AFLP analysis to evaluate genetic relationships in poinsettia plants.

The Sukhwinder et al. reference discusses the use of AFLP to compare fifteen varieties of rice. Sukhwinder et al. took eight varieties of commercially cultivated rice and seven varieties of wild rice, performed AFLP analysis, and created a dendogram based on the varieties' fingerprints.

The use of AFLP analysis in rice is distinct from its application to poinsettias. Rice, a sexually reproducing crop, has a broad genetic pool, and the study of rice genetics is quite advanced. In contrast, only a limited number of molecular studies have been performed with floral crops. *See* Elizabeth J. Parks and James W. Moyer, *Evaluation of AFLP in Poinsettia: Polymorphism Selection, Analysis, and Cultivar Identification,* p. 4 (copy enclosed). Studying the genetics of asexually reproducing ornamentals such as poinsettias is wrought with challenges very different from that of rice, due in part to the narrow gene pool common to ornamental

asexually reproducing plants. Further, similar to other ornamentals, there has been only rudimentary genetic characterization of poinsettias. AFLP analysis has only recently been used on ornamentals at all, and much of the genetic fingerprinting in ornamentals has been performed using techniques such as RFLP, RAPD, microsatellites, DAF, and ASAP. *See* Parks and Moyer at 4-5. As such, at the time of invention, the use of AFLP to study the genetic fingerprint of poinsettias was not obvious, and no suggestion or motivation is found in Sukhwinder et al. or in any of the other cited references.

Further, one of ordinary skill in the art at the time of invention would not have had any reasonable expectation that AFLP analysis could be applied to poinsettia. As discussed above, poinsettias are asexually propagated plants, with a narrow gene pool and very little genetic characterization. There could have been no reasonable expectation for the ordinarily skilled worker prior to the investigations carried out by the present inventors, and disclosed in the instant application, that one would have been able to apply AFLP analysis to determine genetic relationships among poinsettia cultivars.

The deficiencies of the primary reference are not remedied by Ling et al., Sukhwinder et al., or Dice, taken alone or in any combination. Applicants respectfully emphasize that the Ling et al. reference is lacking in several important respects as compared with the presently claimed invention. First, Ling et al. only looked at nine poinsettia cultivars, which were from widely differing groups. *See* Parks and Moyer at p. 5. Additionally, Ling et al. failed to assess the variability of (*i.e.*, validate) each polymorphism to show that each polymorphism was reproducibly and consistently linked to cultivar identity. Park and Moyer, p. 5. Thus, Ling et al. was able to distinguish a small number of genetically diverse cultivars using RAPD analysis, but does not disclose or suggest a method of determining genetic relationship or differences between a large number of genetically similar cultivars, as was achieved by the present inventors.

There are approximately 175 cultivars of poinsettia, and the inventors have used the claimed methods to define the fingerprints of 104 of the 175 cultivars. This is roughly 60% of the total cultivars, as opposed to Ling et al, who only looked at

about .05% of the total poinsettia cultivars. Additionally, the AFLP polymorphisms identified by the inventors are able to differentiate all but 21 pairs of cultivars out of 3240 pairwise comparisons of 81 cultivars. *See* Parks and Moyer at p. 14. This far surpasses the scope of what was explored in Ling et al., where relatively few cultivars were compared. Moreover, it would not have been obvious from Ling et al., taken alone or in combination with Sukhwinder et al., that such a high degree of resolution among poinsettia cultivars as achieved by the present invention would have been possible.

In addition, the application of AFLP analysis to poinsettia was not straightforward. One of ordinary skill at the time of invention would have expected that poinsettia, as an asexually propagated plant, would have essentially no intracultivar variability. In fact, as described in the present application (specification p. 7, lines 17-25; p. 25, lines 10-16), the inventors validated each polymorphism and eliminated those that were related to intracultivar variability, thereby vastly improving the resolution of the fingerprinting analysis as compared with Ling et al. The present invention provides a method of determining genetic relationships and differences between poinsettia plants using polymorphisms that are reproducibly linked to cultivar identity. The problem of intracultivar variability was not appreciated by Ling et al, and neither Ling et al, Sukhwinder et al., nor Dice, alone or in any combination, disclose or suggest that it would be possible to overcome the interfering effects of intracultivar variability or teach a method for doing so as was achieved by the present invention.

As stated by Parks and Moyer (p. 16), "[o]ther AFLP studies to date, especially those in ornamentals, have not addressed intracultivar variation of polymorphisms." In contrast, the inventors analyzed the intracultivar variation of AFLP polymorphisms to determine the degree of variation in the species. The inventors used intracultivar variation to identify AFLP polymorphisms from highly variable regions of the genome and to eliminate these polymorphisms from use in fingerprint analysis. The inventors' work "shows that analysis of the intracultivar variation of AFLP polymorphisms is vital to determine the degree of variation in the species of interest." Park and Moyer at p. 16. Prior AFLP studies have focused on

scoring a large number of polymorphisms to explain expected relatedness. Parks and Moyer at p. 15-16. The present invention differs in that it focuses on the quality of polymorphisms rather than just the quantity. As such, the present invention provides for "selection of appropriate polymorphisms through validation [which] leads to a robust system for analysis of relationships and creation of an AFLP fingerprint." Parks and Moyer at p. 16.

Claims 3-11 recite a "method of estimating a genetic relationship of a first poinsettia plant to a poinsettia plant that is a representative member of a specific breeding family." One of the significant advances of the present invention is the discovery that the genetic fingerprint of poinsettia cultivars using cultivar-linked polymorphisms correlates with breeding history such that fingerprinting techniques can be used to reliably distinguish breeding families. Thus, one of the advantages of the present invention is that it provides a way of evaluating whether a poinsettia cultivar is "essentially derived" from another poinsettia cultivar. It was not at all obvious at the time of invention that polymorphisms could be identified that not only indicate cultivar similarity or differences, but also track the breeding history or pedigree of the plant.

As described above, poinsettia is an asexually reproducing species, with only limited genetic information available. Until recently, most poinsettia breeding was carried out on a relatively informal basis, frequently by hobbyists, without rigorous documentation of breeding history. Further, like many asexually propagated species, new poinsettia cultivars are frequently identified by the selection of naturally occurring or induced mutations rather than by breeding techniques. As a result, there is very little pedigree information available for poinsettias as compared with crop plants such as corn or rice.

It was not at all obvious prior to the investigations carried out by the inventors that the polymorphisms and genetic fingerprints would be powerful enough to track the selection/breeding history of a broad range of poinsettia cultivars and that the different breeding families would have distinct and closely related fingerprints (*see, e.g.*, the clustering of breeding families in the dendrogram of Figure 1 of the application). In other words, it would not have been obvious that the differences in

the fingerprints would be related to breeding family or breeding history. Certainly, none of the cited references, alone or in any combination, disclose or suggest that genetic fingerprinting can be used to identify breeding history. As described above, the Ling et al. reference used poinsettia cultivars from very different families and did not in any way evaluate whether genetic fingerprinting correlates with pedigree.

In view of the foregoing, Applicants respectfully submit that the suggestion and reasonable expectation of success for the present invention cannot be found in Ling et al., Sukhwinder, et al, and Dice. Therefore, Applicants respectfully request that the outstanding rejection of claims 1-7, 10, 11, 21-24, 27-30, 63, 64, and 69 under 35 U.S.C. § 103(a) as being unpatentable over Ling et al., in view of Sukhwinder et al., as defined by Dice, alone or in any combination, be withdrawn.

ii. Rejections Over Ling et al., In View Of Barker et al., as Defined by Tullos

In rejecting Claims 1-6, 10, 11, 21-23, 27-30, 63, 64, and 69, the Office Action states that "[i]t would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention was made to improve the poinsettia cultivar genetic analysis method of Ling et al. and further modify the RAPD procedure of Ling et al. to the improved method of cultivar analysis using AFLP techniques as per the teachings of Barker et al." The Applicants respectfully disagree.

The Ling et al. reference has been discussed at length above. The deficiencies of Ling et al. are not remedied by Barker et al. or Tullos. The Barker et al. reference is directed to the use of RAPD and AFLP analyses to characterize genetic diversity in 19 willow cultivars. As with the Sukhwinder et al. reference discussed above, Barker et al. is distinguishable from the work of the present inventors. Again, the willow, a crop plant, is distinguishable from an ornamental plant such as poinsettia. As discussed in the preceding section, poinsettia is an asexually propagated ornamental plant, and little genetic information is available regarding this species. The work of Barker et al. with willow cannot render the application of AFLP for genetic analysis of poinsettia obvious.

Applicants further reiterate that the AFLP analysis performed by the inventors is distinguishable from the AFLP analysis performed by Barker et al. Prior AFLP studies to date, especially those in ornamentals, have not addressed intracultivar variation of polymorphisms. *Id.* at 16. In contrast, the present inventors analyzed the intracultivar variation of AFLP polymorphisms to determine the degree of variation in the species. The inventors used intracultivar variation to identify AFLP polymorphisms from highly variable regions of the genome and eliminate these polymorphisms from use in fingerprint analysis. Thus, the invention provides high-resolution methods for evaluating genetic relationships in poinsettia using polymorphisms that are reproducibly linked to cultivar identity.

In view of the foregoing discussion, Applicants submit that the cited art provides no motivation to one of ordinary skill in the art with respect to the present invention. Therefore, Applicants respectfully request that the outstanding rejection of claims 1-6, 10, 11, 21-23, 27-30, 63, 64, and 69 under 35 U.S.C. § 103(a) as being unpatentable over Ling et al., in view of Barker et al., as defined by Tullos be withdrawn.

iii. Claims 2, 4-7, 10-11, 22-24, 27-30, and 64.

Applicants submit that Claims 2, 4-7, 10-11, 22-24, 27-30, and 64 are nonobovious over the cited references for all the reasons addressed in the preceding two sections. Applicants additionally note that the Office Action recognized the novelty and nonobviousness of the elected combination of DNA sequences. (Office Action ¶ 20). As Claims 2, 4-7, 10-11, 22-24, 27-30, and 64 recite the elected combination of sequences, Applicants respectfully submit that the subject matter of these claims can be further distinguished on that basis. Therefore, Applicants respectfully request that the outstanding rejection of claims 2, 4-7, 10-11, 22-24, 27-30, and 64 under 35 U.S.C. § 103(a) as being unpatentable over Ling et al. in view of Sukhwinder et al., as defined by Dice and over Ling et al. in view of Barker et al., as defined by Tullos be withdrawn.

III. Conclusion.

The concerns of the Examiner having been addressed in full, Applicant respectfully requests withdrawal of all outstanding rejections and the issuance of a Notice of Allowance forthwith. The Examiner is encouraged to address any questions regarding the foregoing to the undersigned attorney, who may be reached at (919) 854-1400.

Respectfully submitted,

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Attachment: Parks and Moyer manuscript

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Evaluation of AFLP in Poinsettia: Polymorphism Selection, Analysis, and Cultivar Identification

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Subject Category: Molecular Biology-Biotechnology

Evaluation of AFLP in Poinsettia: Polymorphism Selection, Analysis, and Cultivar Identification

Additional Index Words. DNA, floral crop, intracultivar variation, fingerprinting, genetic analysis, molecular differentiation

Abstract. Fingerprinting using molecular markers is a highly effective method of cultivar identification that is a powerful aid to traditional methods based on morphology. Amplified Fragment Length Polymorphism (AFLP) is a robust and reliable method for generating molecular markers that has been used to evaluate many crops for a variety of applications. In this study, AFLP was used to develop and validate robust genetic fingerprints for poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzch) cultivars. Polymorphism selection was completed to facilitate the identification of useful polymorphisms and minimize future fingerprinting costs and time. Poinsettia is a highly variable crop subject to genetic drift and variable cultivars. Validation of polymorphisms to remove those associated with intracultivar variation improved the reliability of the fingerprinting. The result was a poinsettia AFLP database that defines the genetic fingerprints of 104 cultivars. Cluster analysis illustrated differentiation of most poinsettia cultivars tested. Selection of a subset of AFLP polymorphisms resulted in clustering of cultivars according to known origin and breeding program. This method has applications not only for cultivar identification for cultivar protection, and maintenance of cultivar

uniformity, but also has the potential application of developing markers for important traits.

Poinsettia is the most valuable potted plant in the United States, with a wholesale value of $256 million in 2001 (USDA, 2003). Poinsettias were introduced to the US in early 1800's, yet the first commercial quality cultivars were not introduced until 1963. Today, more than 175 cultivars of cultivated poinsettia are available. Because of the ever-changing selection of cultivars and their valuable market share, breeders are under intense pressure to develop new cultivars. With this process comes the desire to protect the cultivars and breeder's rights. Evaluation of morphological characteristics such as bract color, growth habit, and time to flower has been the primary method of cultivar identification; however, there are several shortcomings to this method. Morphological characteristics may be similar between some cultivars, making differentiation difficult. These traits are also influenced by environmental conditions, which can cause variation in their appearance. Finally morphological evaluation can be costly, as plants must be maintained for an entire growth cycle to score many of these traits.

Molecular techniques have distinct advantages over morphological evaluation for cultivar identification. They are not influenced by environmental factors, making them more reliable and stable. These methods can be applied at almost any stage of growth, reducing the time and cost of cultivar identification. Molecular techniques can provide more genetic information, since the number of molecular markers is virtually unlimited. A limited number of molecular studies have been done with floral crops. Fingerprinting of petunia (*Petunia hybrida* L.) has been reported using restriction fragment length polymorphism (RFLP) (Beyermann et al., 1992; Vainstein and Ben-Meir, 1994) and randomly amplified polymorphic DNA (RAPD) (Peltier et al., 1994). Microsatellites have been used for molecular analysis of rose (*Rosa hybrida* L.) (Esselink et al., 2003).

Recently, methods such as randomly amplified polymorphic DNA (RAPD) (Ling et al., 1997), DNA amplification fingerprinting (DAF) (Starman and Abbitt, 1997), and arbitrary signatures from amplification profiles (ASAP) (Starman et al., 1999) have been applied to poinsettia to provide faster and more definitive methods of cultivar differentiation. However, these studies included a limited number of cultivars (9, 11, and 11, respectively) and did not assess the variability of (validate) each polymorphism nor was the method tested across diverse as well as closely related cultivars.

Amplified fragment length polymorphism (AFLP) has distinct advantages over other molecular techniques. AFLP detects polymorphisms throughout the genome by selective amplification of restriction fragments, rapidly producing a large number of markers. AFLP is highly reproducible (Jones et al., 1997) and requires no prior genetic information (Vos et al., 1995). Exploitation of the AFLP technology could provide increased resolution capable of differentiating the more than 175 existing poinsettia cultivars. In addition, this database could be used to generate estimates of similarity between candidate and existing cultivars.

Research with other crops has shown the utility of AFLP for determining relationships between cultivars and for cultivar identification, including bermudagrass (*Cynodon* L.C. Rich.) (L.H. Zhang et al., 1999), eggplant (*Solanum* L.) (Mace et al., 1999), and lettuce (*Lactuca* L.) (Hill et al., 1996). Recently, AFLP has been applied to ornamental crops for the same purpose, including geranium (*Pelargonium* L'Hér.) (Barcaccia et al., 1999), peruvian lily (*Alstromeria* L.) (Han et al., 2000; Han et al., 1999), rose (*Rosa* L.) (D. Zhang et al., 1999), daylily (*Hemerocallis* L.) (Tomkins et al., 2001), and new guinea impatiens (*Impatiens hawkeri* W. Bull.) (Carr et al., 2003; J.H.

Lyerly, unpublished data). Generally, these studies show that genetic marker data agrees with available pedigree data (Carr et al., 2003).

The objectives of this study were to optimize the use of AFLP as a fingerprinting tool for poinsettia and to determine the extent of variability detected with specific AFLP polymorphisms. In this study we have used AFLP to identify a set of polymorphic DNA fragments that are useful for poinsettia cultivar differentiation. The polymorphisms were selected based on their consistent presence or absence in replicates of selected cultivars. In addition multiple statistical models were evaluated for use in generating similarity or dissimilarity indices that would facilitate genotype comparisons.

Materials and Methods

Plant material. One hundred four poinsettia genotypes and two other *Euphorbia* L. species, *E. fulgens* Karw. ex Klotzch and *E. cornastra* (Dressler.) Radcl.-Sm., were included in the fingerprint analysis. For the validation study, 77 sources of nine genotypes, as shown in Table 1, were collected from locations worldwide and coded to remove analytical bias. Plant material was provided by breeders or collected from two poinsettia trials conducted at the Horticulture Field Laboratory, NCSU.

Genomic DNA was isolated from fresh tissue using a modified benzyl chloride procedure (Zhu et al, 1993). The extraction protocol was adapted to small volume processing of 150 mg of leaf tissue in a microcentrifuge tube. Fully expanded leaves with midribs removed were selected from all cultivars for extraction.

AFLP analysis. The AFLP protocol was performed as described by Vos, *et al.* (1995) using AFLP Analysis System I (Life Technologies, Gaithersburg, Md.) with the following modifications. The Life Technologies protocol was modified by extending the restriction digest incubation to overnight and increasing the ligation incubation to 6 hours at 16°C to improve reproducibility in the final AFLP result. ^{33}P was used to label the *Eco* RI primer for specific amplification. The products of the specific amplification were electrophoresed on a 6% denaturing acrylamide gel at 60 W for approximately 2 hr. The gel was fixed in 5% acetic acid/5% methanol and dried in a gel dryer (BioRad, Hercules, Calif.), then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, Calif.) overnight. The screen was then scanned on a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) and the gel image was saved as a TIFF file.

Data analysis. The gel image was analyzed using Pro-RFLP image analysis software (DNA ProScan, Nashville, Tenn.). ϕ-X174/*Hinf* I (Promega, Madison, Wis.) was used as the standard reference to size the AFLP fragments. Selected AFLP polymorphisms were sized and scored as present (1) or absent (0). The scored data was exported as 1 or 0 to a Microsoft Excel (Microsoft, Inc, Seattle, Wash.) spreadsheet. Coefficients of association, for both similarity and distance, were generated from the binary data using five different models, three that incorporate only positive matches (1/1), and two that incorporate both positive and negative matches (0/0). Distance was calculated using a model described by Lynch (1988). Similarity and all other analyses were calculated using four different coefficients in the statistical software package NTSYSpc 2.0 (Exeter Software, Setauket, N.Y.): (1) Dice, $S_{ij}=2a/(2a+b+c)$, where S_{ij} is the similarity between two cultivars, i and j, a is the number of fragments shared by i and j, b is the number of bands present in i and

absent in j, and c is the number of bands present in j and absent in i (Dice, 1945); (2) Jaccard, S_{ij}=a/(a+b+c) (Jaccard, 1908); (3) simple matching, S_{ij}= a+d/a+b+c+d, where d is the number of bands absent in both i and j (Sokal and Michener 1958); and (4) unnamed coefficient 1, S_{ij}=2(a+d)/2a+b+c+2d. The similarity or distance matrices were then analyzed using four different SAHN clustering methods, UPGMA (unweighted pair-group method; Sokal and Michener, 1958), WPGMA (weighted pair-group method; Sneath and Sokal, 1973), CL (complete linkage; Lance and Williams, 1967), and SL (single linkage; Lance and Williams, 1967). Dendrograms were created from the clustered matrix using TREE. The COPH and MXCOMP programs calculated the goodness of fit of the clustering to the data matrix. Principal coordinates analysis was performed using DCENTER and EIGEN.

Results

Selection of polymorphisms. A two-level screening strategy was used to determine which AFLP primer pairs were the most appropriate for fingerprinting poinsettia cultivars. Initially, all 64 possible primer combinations in AFLP Analysis System I were used to amplify DNA from four poinsettia genotypes (C1, C17, C27, and Selection 119) that were the non-grafted progenitors of three major cultivar groups. The phytoplasma-free genotypes were selected to insure that the polymorphisms were poinsettia in origin, and to span the diversity of poinsettia cultivars. Primer combinations were ranked based on the number, intensity, and reproducibility of polymorphisms. Polymorphisms were selected for analysis if they were present in at least one phytoplasma-free cultivar, easily scored on the AFLP image in terms of intensity and separation from other fragments, and reproducible in at least two independent amplifications. A preliminary evaluation of the

four best primer combinations on a larger number of cultivars, listed as 1-4 in Table 2, did not result in a high level of differentiation.

A second evaluation of the 30 best primer combinations from the initial screen was completed with a set of 12 cultivars spanning a broad range of similarities, including three sources of the same cultivar from different sources for detecting intracultivar variation, listed as Group 1 in Table 1. The primer combinations that generated the most useful polymorphisms were scored and analyzed individually, and prioritized according to their ability to detect polymorphisms between closely related cultivars without detecting intracultivar variation. The new primers were then analyzed in combinations with the original four primers according to priority, until the similarities for the set of cultivars were optimized; this balanced differentiation of cultivars with clustering of related cultivars. Four additional primer combinations were selected, listed as 5-8 in Table 2. A total of 98 AFLP polymorphisms were selected for further analysis, resulting in Jaccard similarities ranging from 0.27 to 0.98.

Reproducibility of polymorphisms. To further validate the reproducibility and reliability of the AFLP fingerprints, the polymorphisms were evaluated. In a preliminary study to test the hypothesis that some polymorphisms were in regions of the genome associated with intracultivar variation, we analyzed plants from multiple sources of five cultivars: 'Freedom Red' (7 sources), 'Hegg Dark Red' (2 sources), 'Peterstar Red' (2 sources), 'Lilo Red' (2 sources), and 'V14 Glory Red' (1 source). The results of this test demonstrated the presence of intracultivar variation among the polymorphisms.

In a larger test designed to identify the polymorphisms that should be excluded from the analysis, we used 77 sources of nine different cultivars as listed in Table 1. The

amount of intracultivar variation of the polymorphisms was different for each of the cultivars. 'Angelika Red' showed the least variation with differences noted in 10 of the 98 polymorphisms. Sources from four of the validation cultivars, 'Freedom Red', 'V14 Glory Red', 'Snowcap', and 'Hegg Dark Red', showed the most variation. One source of each of these cultivars was very different from all other sources of the cultivar; differences were seen in as many as 33 of the 98 polymorphisms. Polymorphisms that were variable among sources of a given cultivar were likely to be variable among sources of one or more other cultivars; 22 varied in one cultivar, and 35 varied in more than one. Of the 98 polymorphisms, eight were consistent in all sources of all cultivars. In total, 57 of the 98 polymorphisms were found to be highly variable, varying in more than one source of a particular cultivar or cultivars. These highly variable polymorphisms were eliminated from AFLP analysis. The 41 validated polymorphisms were used to create a poinsettia AFLP database for poinsettia genotypes and 2 outgroup species, *E. cornastra* and *E. fulgens*. This database includes 81 commercially released cultivars as well as 23 unnamed, unreleased genotypes of poinsettia.

Data analysis. To determine which statistical methods would yield the most accurate representation of the relationships between the cultivars in this study, comparisons were made between five similarity or distance coefficients and four clustering methods. The dendrograms constructed using the various association and clustering methods were examined and the cophenetic correlation coefficients of each were compared (Table 3), to test the goodness of fit of the association coefficient to its respective dendrogram. The association coefficients that gave the highest cophenetic correlation coefficients were those that incorporated only positive matches (1/1). UPGMA clustering gave the highest

correlation coefficients of the clustering methods, from 0.813 to 0.877, indicating a good fit of the similarity matrix to the dendrogram. The coefficients that incorporated only positive matches, Dice, Lynch, and Jaccard, yielded the same cultivar group clusters in the same orientation as shown in the dendrogram of 81 named cultivars and 2 outgroup species (Fig. 1). The two coefficients that incorporated positive and negative matches, SM and UN1, also clustered the cultivar groups, but the orientation of the clusters on the dendrogram differed from that of the other three methods. Jaccard's similarity coefficient, when clustered with the UPGMA technique, gave the highest of all the correlation values, 0.877, indicating the best fit to the data.

There was a full range of Jaccard similarity coefficients. Between poinsettia cultivars, similarity coefficients ranged from 1 (identical), between 21 different cultivar pairs, and 0.219 (least similar), between 'Airbrush' and 'Freedom Rose'. The similarities between related cultivars ranged from 1 to 0.615, with the lowest similarity being between 'Freedom Red' and its color sport 'Freedom Rose'. *E. conrastra* had much lower similarities to the poinsettia cultivars, with coefficients ranging from 0.032 to 0.217. *E. fulgens* also had lower similarities to the poinsettia cultivars, ranging from 0.152 to 0.433. The similarity between these two outgroup species was 0.095.

A small set of the cultivar pairs could not be differentiated with this set of AFLP polymorphisms. The 21 identical pairwise comparisons involved 16 cultivars from three main cultivar groups; one comparison involved 'Lilo', 7 were from 'Freedom', and 13 were from 'Angelika'/ 'Peterstar'. All of the cultivar pairs with identical coefficients were related; some of these cultivars are color sports resulting from either natural or induced mutations. Seven comparisons involved color variants of a cultivar and seven

others were color variants of related cultivars. Four comparisons involved cultivars with a leaf variegation mutation. One comparison was between a cultivar and a selection of the same cultivar. The remaining two comparisons were also pairs of derivative cultivars, and had similar coloring of bracts and leaves.

Figure 1 shows the dendrogram of the 81 commercially released cultivars and the two *Euphorbia* outgroup species. A critical value of approximately 0.63 separated the dendrogram into 5 distinct clusters: 'Lilo', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'. The most distant poinsettia cultivar was 'Xenia Red Deluxe', which branched from the dendrogram at approximately 0.42. *E. fulgens* and *E. cornastra* made up the outermost branches at approximately 0.31 and 0.12, respectively.

Principle coordinates analysis gave further support to the clusters created by UPGMA clustering. The first three eigenvectors explain 56.93%, 9.88%, and 4.09% of the total variation, cumulatively accounting for 70.91% of the variation. The three-dimensional PCO plot shows the same clusters found with SAHN analysis, 'Lilo', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'.

Cultivar group analysis. The genetic lineages were not available to provide direct evidence of a correlation between polymorphic profile similarity and genealogy; furthermore, many cultivars are derived from natural or induced mutations arising from existing genotypes. Alternatively, an iterative strategy was derived to determine the extent that the measure of similarity of polymorphic profiles reflected genetic relationships and thus the probability that a cultivar originated from a specific breeding program. The strategy consisted of obtaining genetic information from available plant

patents and grouping cultivars based on known origin and breeding program. A subset of 14 of the 41 polymorphisms present in all seven of the Freedom-derived cultivars was initially selected as the basis of a strategy to identify the breeding program or origin of the genotypes. When this set of polymorphisms was used to generate a dendrogram, the Freedom-derived cultivars clustered together with a similarity of 1, with other cultivar groups clustering in a similar manner to that on the dendrogram that included all of the AFLP polymorphisms. Examination of nine cultivar groups revealed within-group consistency of five of the 14 polymorphisms; within the V14 Glory, Freedom, Celebrate, Cortez, Sonora, Peterstar, Angelika, Lilo, and Hegg cultivar groups, the polymorphisms were either consistently present or absent. In addition, two more that were absent in all Freedom cultivars were consistently present or absent in the other nine cultivar groups. Using these 7 polymorphisms, a new dendrogram was generated, which placed all of the genotypes sharing a common origin into clusters with similarities of 1 (Figure 2). The cophenetic correlation of this dendrogram was 0.869, showing high correlation of the clustering to the data. Principal coordinates analysis supported the same clusters, with the first three eigenvectors explaining a total of 68.67% of the variation.

To test the significance of the subset of AFLP polymorphisms, a random set of 7 polymorphisms was generated from the 41, similarities were calculated, and a dendrogram was generated. The majority of cultivar groups were only partially clustered, and clustering of completely unrelated cultivars occurred with this set of polymorphisms. This shows the selection of the set of AFLP polymorphisms for cultivar group analysis was not due to random chance; clustering was dependent on the particular

polymorphisms and was not an artifact of the small number of polymorphisms used in the analysis.

Discussion

The dendrogram of 81 commercially released cultivars constructed using the entire set of 41 validated polymorphisms is consistent with known pedigrees of poinsettia according to breeder and cultivar patent information. Hegg, Lilo, and Splendor cultivars share a common ancestry, and they form a unique cluster on the dendrogram. 'Cortez' resulted from a cross of 'Lilo' and an unknown cultivar, and it occupies a cluster adjacent to 'Lilo'. 'Malibu Red' was developed from a cross involving 'Cortez', and clusters closely to it. Mutation or natural breeding from 'Angelika Red' developed a number of cultivars that cluster in the center of the dendrogram. The three Celebrate cultivars form the next cluster on the dendrogram. Cultivars that share a background of 'V-14', 'Jingle Bells' and 'Monet' form a distinct cluster. The 'Freedom' cluster includes the 'Freedom Red' color sports developed by mutation and natural breeding, as well as 'Coco Red', 'Festival Red', and 'Pepride', all developed from 'Freedom Red'. Finally, the outgroup species *E. fulgens* and *E. cornsatra* occupy the outmost branches of the dendrogram.

The set of 41 AFLP polymorphisms was able to differentiate all but 21 pairs of cultivars out of 3240 pairwise comparisons of the 81 cultivars. Although all cultivars appeared to be differentiated using the full set of 98 AFLP polymorphisms, removal of polymorphisms that were hypervariable reduced the resolution of the test, causing the 21 pairs of cultivars to no longer be differentiated. All but two of the cultivars that could not be differentiated with the set of polymorphisms are easily separated by morphological traits; Nutcracker Red and Peterstar Red were the only morphologically similar cultivars

that could not be differentiated with this set of AFLP data. All other undifferentiated cultivars were either color sports of one another or had different leaf variegation. Most of those cultivars that are the most difficult to distinguish morphologically, such as the numerous red, white, and pink cultivars in the study, were differentiated with AFLP fingerprinting. This demonstrates the importance of validating polymorphisms, as some that appear to discriminate between sports of some poinsettia cultivars could potentially cause false identification of a cultivar if used in fingerprinting. Sports may be difficult to distinguish genetically (Weising et al, 1995), and a more sensitive method such as microsatellites may be necessary to fully differentiate the cultivars.

Additional selection of polymorphisms facilitated reliable classification of the cultivars into clusters indicative of genetic background and the breeding program or origin of the cultivar. The core set of seven AFLP polymorphisms selected using color variants places the cultivars in tight clusters of cultivar groups. Obvious cultivar groups with shared names clustered as expected, into groups with a similarity of 1. Other groups on the dendrogram with a similarity of 1 appear to cluster several cultivar groups together, 'Angelika' and 'Peterstar' groups for example. Further investigation into the breeding history of these cultivar groups revealed common ancestry; clustering using the core set of polymorphisms was a good predictor of breeding history of the cultivars. This cultivar group AFLP analysis could be used as a preliminary identification tool to place cultivars in the correct cultivar group, or it could be used to identify breeding group for cultivar protection purposes.

Other AFLP studies (Carr et al. 2003; Han et al., 2000) have focused on scoring a large number of polymorphisms to explain expected relatedness. However, this study

shows that it is the quality as well as quantity of information that the polymorphisms contain, and not the quantity of polymorphisms themselves, which explains relationships and creates AFLP fingerprints. Selection of appropriate polymorphisms through validation leads to a robust system for analysis of relationships and creation of an AFLP fingerprint. The process is similar to validation of polymorphisms for any set of complex traits; here it is cultivar identification. Optimization of the set of polymorphisms can provide any level of differentiation that is needed. To help determine the breeding origin of a cultivar, the group-specific data can be used, and to differentiate, or fingerprint, a particular cultivar, the entire set of polymorphisms should be used. In addition, minimizing the number of primer combinations reduces the cost and time required to fingerprint cultivars. A high percentage of the expected relatedness can be explained by selecting the optimal primer combinations. In one other example, Ellis *et al.* (1997) found that by selecting the six best primer combinations, more than 80% of the expected relatedness could be explained. This study selected the best 8 primer combinations from 64 possible, so it is possible to conclude that this study has found the majority of expected relatedness.

Other AFLP studies to date, especially those in ornamentals, have not addressed intracultivar variation of polymorphisms (Carr et al. 2003; Ling et al. 1997; Starman and Abbitt 1997; Starman et al. 1999; D. Zhang et al. 1999). However, this study shows that analysis of the intracultivar variation of AFLP polymorphisms is vital to determine the degree of variation in the species of interest. Poinsettia is a vegetatively propagated crop that must be selected annually to maintain crop uniformity, demonstrating the variability that exists in this crop. Once the degree of intracultivar variation is established, some

method to compensate for it should be implemented to establish a more robust fingerprint. The variation of specific AFLP polymorphisms in several different cultivars suggests that some of them likely originate from highly variable regions of the genome, and should be eliminated from fingerprint analysis. Discriminating between those polymorphisms that are reflective of this heterogeneity and those that are stable and connected with the distinct nature of that cultivar is essential to generating a reliable fingerprint. In addition, the high degree of intracultivar AFLP variation in poinsettia suggests that a molecular tool such as AFLP would be valuable in maintaining homogeneity of cultivar when used for marker-assisted breeding.

An additional important factor that determines reproducibility is complete restriction digestion. Many floral crops, including poinsettia, require optimization of extraction and digestion protocols for preparation of high-quality DNA requisite for reproducible polymorphic profiles (Barcaccia et al. 1999; Carr et al. 2003; J.H. Lyerly, unpublished data). Incomplete digestion can be detected in the AFLP pattern by loss of small AFLP fragments, along with gain of larger fragments (Life Technologies, Gaithersburg, Md.). Therefore, commonly occurring monomorphic fragments in the AFLP pattern, particularly those 100 bp and smaller, can serve as controls for complete restriction digest. Reliability and reproducibility of the AFLP technique is additionally insured by the stringent primer annealing conditions known as "touch-down PCR", which minimizes mispriming, and thus greatly reduces aberrant PCR products.

The repeatability of the AFLP banding patterns coupled with the validation of the polymorphisms by testing of multiple sources of various cultivars provides credibility to the AFLP fingerprints and the relationships concluded from them. Additional support of

the data comes from the consensus of the different methods of analysis. The similar clustering in the dendrograms generated using different association coefficients and clustering methods verifies that the clusters are distinct (NTSyspc 2.0, Exeter Software, Setauket, N.Y.). Likewise, the clustered groups in the Principal Coordinates Analysis gives further support to the clusters.

Information in this study has shown that validation of polymorphisms is essential for the AFLP technique to be an effective and robust tool for identifying and differentiating poinsettia cultivars, as well as for determining breeding relationships. AFLP analysis of poinsettia and other floral crops provides valuable information that will facilitate the use of molecular methods for cultivar protection, support in breeding programs, and the potential to develop markers for desirable characteristics.

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Table 1. Poinsettia cultivars used to identify polymorphisms useful for fingerprinting and cultivar identification. Group 1 cultivars were used to identify AFLP primers useful for cultivar identification. Group 2 cultivars were used to evaluate intracultivar variation of the polymorphisms and to validate the poinsettia AFLP fingerprints.

Cultivar	Number of sources
Group1 Cultivars	
Bonita	1
Freedom Jingle Bells	1
Freedom White	1
Gross Heirloom	1
Peterstar Jingle Bells	1
Peterstar Pink	1
Maren	1
Angelika Red	1
Freedom Red	3
Peterstar Red	1
Group 2 Cultivars	
Angelika Red	7
Freedom Red	13
Peterstar Red	8
Hegg Dark Red	5
Supjibi	6
Lilo Red	5
Snowcap	8
Sonora Red	19
V-14 Glory Red	6

Table 2. AFLP primer combinations selected for fingerprint analysis of poinsettia cultivars. Average includes the total number of fragments ranging from 60 to 750 bp. Scored polymorphisms are all polymorphisms scored prior to validation. Cultivar-linked polymorphisms are validated polymorphisms used in the final analysis. E= GACTGCGTACCAATTC and M=GATGAGTCCTGAGTAA.

	Primer combination	Average number of fragments	Scored polymorphisms	Cultivar-linked polymorphisms
1	E-AAG/M-CTA	99	10	5
2	E-AAG/M-CTG	91	18	9
3	E-ACA/M-CTC	95	8	4
4	E-ACA/M-CTT	109	10	4
5	E-ACA/M-CTA	88	11	4
6	E-ACA/M-CTG	70	6	2
7	E-AGC/M-CAC	55	21	7
8	E-AGC/M-CTA	71	14	6
Total			98	41

Table 3. Cophenetic correlation coefficients for the five similarity or distance coefficients and four clustering methods applied to the final AFLP data set containing the 81 commercial poinsettia cultivars and 2 outgroup species.

	Dice	Jaccard	SM	UN1	Lynch
UPGMA	0.870	0.877	0.839	0.813	0.867
WPGMA	0.855	0.861	0.807	0.775	0.817
CL	0.759	0.791	0.775	0.733	0.751
SL	0.785	0.778	0.754	0.726	0.781

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